

**IMAGING STUDIES OF TETRA-SULPHONATED PORPHYRIN IN K562 HUMAN
CHRONIC MYELOID LEUKEMIA CELLS DURING PHOTODYNAMIC THERAPY**

Simona-Florentina Pop^{1,2}, Monica Neagu³, Carolina Constantin³ and Rodica-Mariana Ion^{1,2}

¹National Institute for Chemical and Petrochemical Research, ICECHIM, Analytical Department,
202 Splaiul Independentei, Bucharest, Romania; Tel/fax: +4021.316.30.94;

E-mail: popsimfc@yahoo.com, rodica_ion2000@yahoo.co.uk

2) Valahia University, Targoviste, Unirii Blvd., 18-20, Targoviste, Romania

³Victor Babes", National Institute, Immunology Dep., 99-11 Splaiul Independentei,
Bucharest, 050096, Romania; E-mail: imunoc@vbabes.ro

Abstract

Non-invasive monitoring of molecular targets is particularly relevant to photodynamic therapy (PDT), including the delivery of photosensitizer in the treatment site and monitoring of molecular and physiological changes following treatment. Photodynamic therapy consists of the uptake of a photosensitizing dye by tumor tissue and subsequent irradiation of the tumor with light of an appropriate wavelength matched to the absorption spectrum of the photosensitizing dye. Singlet oxygen is accepted as the main mediator of photocytotoxicity in photodynamic therapy, causing irreversible cell damage by oxidation and degradation of (intra)cellular biomembrane structures, but with minimal systemic toxicity. This report presents a study of the in vitro non-uniform intracellular distribution of 5, 10, 15, 20 - tetra (4-sulfophenyl) porphyrin (TS₄PP) on K562 human chronic myeloid leukemia cell line.

Key words: TS₄PP, K562 human chronic myeloid leukemia cell line; Optical imaging; Photodynamic.

1. INTRODUCTION

Photodynamic therapy (PDT) is a relatively new therapeutical approach for cancer treatment, consisting of the combined use of systemically administered photosensitizing substance and local applications of light emitted by lamps or lasers in the presence of oxygen. The technique is based on the preferential accumulation of photosensitizer in tumor tissue with subsequent cytotoxicity mediated by singlet oxygen production. Its therapeutic usefulness is due to selective and rapid tumor necrosis after illumination of the photosensitizer-containing tumor with light (Ion, 2003, Ion 2006, Calin and Pascu, 2006).

The lethal, acute, direct effects of PDT on tumoral cells and even normal cells depend on: type, dose and location of the photosensitizer on/or in the cell, the photodynamic efficiency of the substance in the environment, parameters of irradiation and oxygen transport.

Different photosensitizers have been tested in human tumor tissues, including porphyrins (hematoporphyrin derivative, porfimer sodium, tetra-sodium-meso-tetra-phenyl-porphyrin-sulphonate), phthalocyanines, chlorines, porphycenes, purpurins, eosin, tetracycline, acridine orange and Rhodamine-123. Porfimer sodium has been the preferred drug used as a photosensitizing substance due to its high affinity for the tumor cell and its strong effect under irradiation with light. In spite of this, the application of sodium porfimer is limited due to some criteria:

- Heterogeneous chemical composition, rich in unknown components;
- Low molar absorption coefficient ($3000 \text{ M}^{-1}\text{cm}^{-1}$) in the range of clinical wavelengths (over than 600 nm);
- Precipitation tendency in aqueous solution;
- Skin persistence;
- Low clearance rate associated by a high maintenance in the reticuloendothelial system, liver and so on, and yielding some toxic effects even in the dark.

The studies in this field are focused on new photosensitisers displaying a homogeneous distribution to tumoral area and subsequently optimum properties for PDT.

Porphyrin has been the preferred drug used as a photosensitizing substance due to its high affinity for the tumor cell and its strong cellular effect after light irradiation. Ion and Apostol (2004) show that porphyrins can form a variety of structures from linear head-to-tail or J-aggregates to fractal aggregates grown under different regimes of aggregation, and can exhibit rich photophysical properties. Photophysical properties of TS₄PP porphyrin, as a synthetic one have been studied extensively in vivo and in vitro with an application for photodynamic therapy of cancer. The size and shape of TS₄PP aggregates in solution have been investigated extensively using spectroscopic and light scattering methods. Aggregates can break into their constituent parts which can be monomers or smaller aggregates and this can probably be shown by the change of the relative peak positions.

The aim of this work is to establish the optical properties of 5, 10, 15, 20 - tetra (4-sulfophenyl) porphyrin (TS₄PP) photosensitizer and its intracellular biodistribution in K562 human chronic myeloid leukemia cell line.

2. MATERIALS AND METHODS

2.1 Photosensitizers

5, 10, 15, 20 - tetra (4-sulfophenyl) porphyrin (TS₄PP) is a negatively charged photosensitizer obtained in the lab after already reported standard methods. The molecular structure of porphyrin dye is shown in Fig. 1. and its purity has been checked by X-ray diffractometry, FTIR spectrometry and UV-Vis spectrophotometry. Porphyrin was diluted in phosphate buffer (pH=7.2) at a 10^{-4} M concentration and different concentrations were afterward made in cell culture medium. All chemicals were used without any purification.

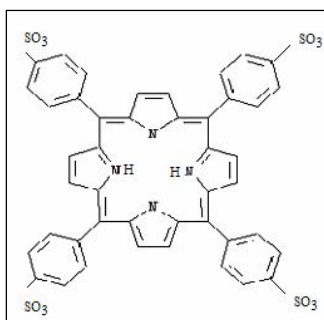


Fig. 1. The structure of TS₄PP (R=SO₃⁻)

All the stock solutions were stored at 4°C in the dark and used in the 14 days interval. As noncellular controls we have used the specific photosensitizer or only DMSO solvent in culture medium at the appropriate concentrations. All the mentioned controls were used to eliminate the possible interferences with the measurement systems on one hand as the photosensitizers have absorption spectra around 500-600nm and on the other hand to subtract the solvent effect, namely DMSO.

2.2. Cell Cultures

K562 lymphoblastic human cell line (supplier ATCC Nr.CCL-243) derived from chronic leukemia was used. Cells were maintained in culture or cryopreserved as stated by the supplier. Briefly, the cells were cultivated in RPMI1640 with phenol red supplemented with 4 mM L-glutamine, 1.5 g/L NaHCO₃ and 10% fetal calf serum (FCS) at 37°C in 5%CO₂ atmosphere at a cell concentration of 1-2x10⁵ cells/mL. In 48-72h cells quadruple their number and the culture medium is changed, cells being reseeded at 1-2x10⁵ cells/mL. All the tests were performed in the exponential growth phase of the cell line (Neagu et al. 2006, Manda et al., 2006). In all the PDT experimental systems the cell line was used in RPMI1640 complete medium without phenol red in order to avoid the interference with the detection methods. Cell viability was assessed by the Trypan blue method.

2.3. Absorption and Fluorescence Spectrophotometry

A Carl Zeiss Jena M400 spectrophotometer was used for absorption spectra registration. Fluorescence spectra were obtained using a Perkin-Elmer LS 50B luminescence spectrometer using excitation wavelengths near the maximum absorbance for each photosensitizer and solvent. All solutions were measured at room temperature in 1 cm or 1 mm pathlength quartz cuvettes. Absorption spectra, fluorescence spectra and fluorescence lifetimes were taken for all solutions using the following concentrations: 1.0 x 10⁻⁷ M, 1.0 x 10⁻⁶ M, 5.0 x 10⁻⁶ M, 1.0 x 10⁻⁵ M, 5.0 x 10⁻⁵ M, 1.0 x 10⁻⁴ M. Stock solutions were prepared in Water:DMSO (99.95%-0.05%) and diluted in Dulbecco's modified Eagle's medium (DMEM) with 1% FBS just before use.

2.4. Photosensitizers loading

The loading was performed at various incubation time (1-48h), cell concentration (0.05 – 1.5 x 10⁶ cells /mL) and photosensitizer concentration (5-250 microg/mL). The loading degree was tested registering the cell suspension's mean fluorescence. Suspensions of loaded cells were thoroughly and quickly washed before imaging and displayed on poly-L-Lysine coated fluorescence treated glass slides. The loading degree was tested by flow-cytometry registering the cell suspension's mean fluorescence and results are presented as index compared to unloaded cells.

2.5. Fluorescence microscopy

Fluorescence microscopy was performed on an optical scatter imaging microscope platform, which was also fitted with an epifluorescence modality, using an inverted microscope (Eclipse T200, Nikon) with epifluorescence. A 10-nm band-pass interference filter placed in the condenser housing yielded an incident red beam centered at $\lambda = 630$ nm (V2A filter).

2.6. Data analysis

All data and images processing was done using Image J software. All images of single cells were extracted from larger images. Each single cell image was processed to visualize and quantify the photosensitizer molecules. For rectangular selections, the x-axis represents the horizontal distance through the selection (Szmackinski et al. 1994).

3. RESULTS AND DISCUSSIONS

3.1. Absorption spectra

(TS₄PP) is an anionic porphyrin, a very large disk-shape molecule which possess four negative charges the sulfonate groups from the four corners. In aqueous solutions, at neutral pH, the electronic absorption spectrum of TS₄PP is typical of free base porphyrins (D_{2h} symmetry) and is characterized by an intense Soret band at around 420 nm and four Q bands in the 500-700 nm range (the aetio-type spectrum).

Aggregation of TS₄PP can be activated by decreasing pH or increasing ionic strength of the solution, and it can be monitored using different positions of absorption peaks corresponding to different species, Fig.2. Could be a zwitterionic between the central diacid group of the sulfonic groups, which could be more responsible for J-aggregation. Because of static Coulombic repulsion, the two central NH fragments are probably distorted out of the aromatic plane. The planar TS₄PP zwitterionic monomer subunit leads to the suggestion of a straightforward structure for both J- and H-aggregates. The electrostatic interaction between the zwitterionic no doubt facilitates this aggregation. The J-aggregate is formed initially as a transparent colloidal solution which is then precipitated from the aqueous solution with time by exposure to light.

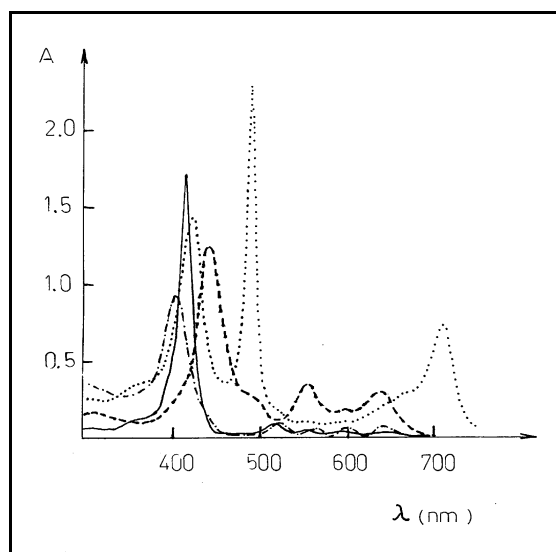


Fig.2. Absorption spectra of different TS₄PP forms:
(-) monomer; (...)dimer; (-.-) H-aggregate; (---) J-aggregate

In acidic medium, new absorption bands (from 490, 707 nm) become dominant when the concentration of TS₄PP exceeds 10⁻⁵ M and they are attributed to the aggregated forms of TS₄PP, Table 1.

Table 1. The specific absorption bands of different TS₄PP forms

<i>Porphyritic forms</i>	<i>B band</i> $\lambda/\epsilon \times 10^3 (\text{nm}/\text{M}^{-1} \cdot \text{cm}^{-1})$	<i>Q bands</i> $\lambda/\epsilon \times 10^3 (\text{nm}/\text{M}^{-1} \cdot \text{cm}^{-1})$
<i>H₂TSPP⁻⁴</i>	412/355	515/130 551/4.5 579/1.9 33/1.01
<i>H₄⁺²TSPP</i>	433/357	550/140 594/3 644/14
<i>H₄²⁺TSPP⁴ J-aggregate</i>	422 490	707
<i>H₄²⁺TSPP⁴ H-aggregate</i>	401	517 552 593 650

Assuming that the distance between molecules is $\sim 5 \text{ \AA}$, the aggregate length can be calculated to be $\sim 10^{-6} \text{ cm}$, showing that the exciton diffusion length is shorter than the aggregate length. This result suggests that the number of molecules per aggregate determined represents the diffusion-limited size of the aggregate. The number of coherently conjugated molecules obtained in this work is in fairly good agreement with previous findings on the aggregation number of TS₄PP obtained. However, it has been proposed that J-aggregates of TS₄PP tend to form large fractal-like structures or macroaggregates in organic solvents. H and J-aggregates of some porphyrins are based on the intermolecular interactions of 3-5 Kcal/mol per porphyrin face. Such aggregates have the size of 5-6 nm in solution. The columnar structure formed by porphyrins has a length of 5 to 27 porphyrin unities (Ion et al. 1998).

3.2. Fluorescence spectra

Useful informations could be obtained also by means of emission spectroscopy. The fluorescence spectrum of the monomer of TS₄PP in Water:DMSO (99.95%-0.05%) shows two peaks at 650 and 700 nm. The fluorescence spectrum of concentrated TS₄PP solution is highly quenched which bring us to the conclusion that the TS₄PP dimer is weakly fluorescent, Table 2.

The TS₄PP emission in Water:DMSO (99.95%-0.05%) strongly depends on the wavelength of excitation. In Water:DMSO (99.95%-0.05%), the emission band of the J-aggregates shifts continuously with the excitation wavelength. The fluorescence of the J-aggregates is very weak compared to the monomeric or H-aggregate, and the first results have been obtained by Frackowiak (1998) and Wrobel (1998).

Table 2. The fluorescence bands of TS₄PP at different concentration in Water:DMSO (99.95%-0.05%)

<i>Porphyrinic form</i>	λ_{exc}	λ_{em}
<i>Monomer</i>	650	700
<i>Dication monomer</i>	672	749
<i>H-aggregate</i>	665	728
<i>J-aggregate</i>	714	724

It is important to predict the type of aggregated structure that probably affects the loading capacity and in the end, the efficiency of PDT. The singlet oxygen formation, consequently the photochemical efficiency is enhanced if face-to-face configurations (H-aggregates) are formed. Because the protonation of the inner nitrogen atoms drastically prevents the incorporation into cells and our porphyrins have good incorporation properties, we presume that this porphyrins exist firstly as H-aggregates, because they are neutral (not at all charged) and stable in solution. This rule could be a logical explanation for the fast application of such porphyrins in biological media in order to delay the J-aggregates formation as precipitate. At the same time, we could presume that H-aggregates exist concomitantly with the J-aggregates. The precipitate is insoluble in water but soluble in DMSO wherein the aggregate dissociates into monomeric porphyrin.

Ion (1998) and Frackowiak (2001) showed that the efficiency of the incorporation of these dyes into cellular membrane changes in the same manner as the singlet oxygen generation. The sulfonated porphyrins were better incorporated (unlike to the non-sulphonated ones). The finding can suggest that aggregated forms (J-aggregates) generated for the porphyrins in Water:DMSO (99.95%-0.05%) are better penetrating the membranes and once in the cellular membrane, dyes are deaggregated by the interaction with lipids and exhibit photodynamic efficiency as the monomeric form.

3.2. Microscopic studies

In the light of changes observed under fluorescence microscopy (fig 3) further studies were undertaken for detailed analysis of time loading of TS₄PP for K562 cells treated with 50 microg/mL TS₄PP (fig. 4).

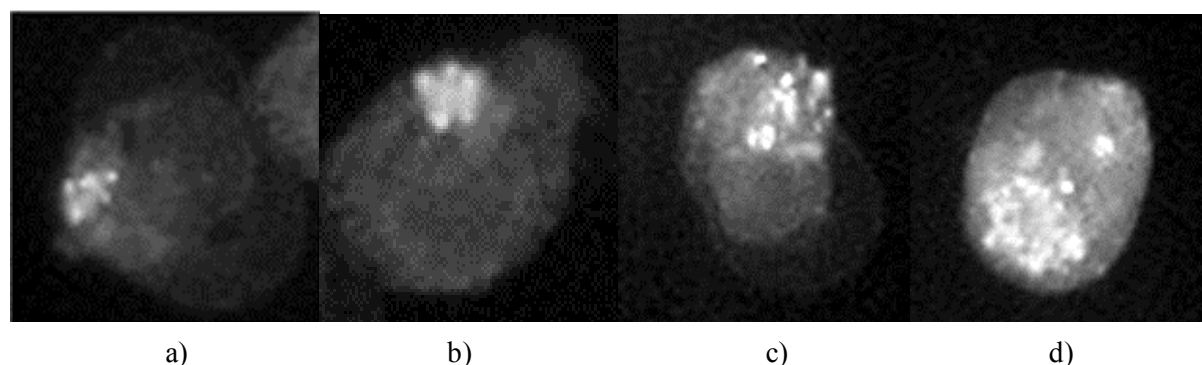


Fig. 3 Fluorescence images of single cell K562 lymphoblastic human line extracted from the integrale images collected with CCD camera, at different incubation time of 50 microg/mL TS₄PP;

a) 2h; b) 4 h; c) 8 h si d) 24 h

After 1 h of TS₄PP administration, the sensitizer shows a non-uniform distribution, the loading area being 45,29 % from cell surface (fig 4.a). After 4h of administration, a sensitizer localisation is observed (fig.4.b) in some cellular targets and an increased fluorescence intensity (fig. 5) is being detected. After 8 h and 24 h post-administration, the sensitizer is released from the cells, (fig.4. c-d), being recognized only in 5,79 % from total cell area.

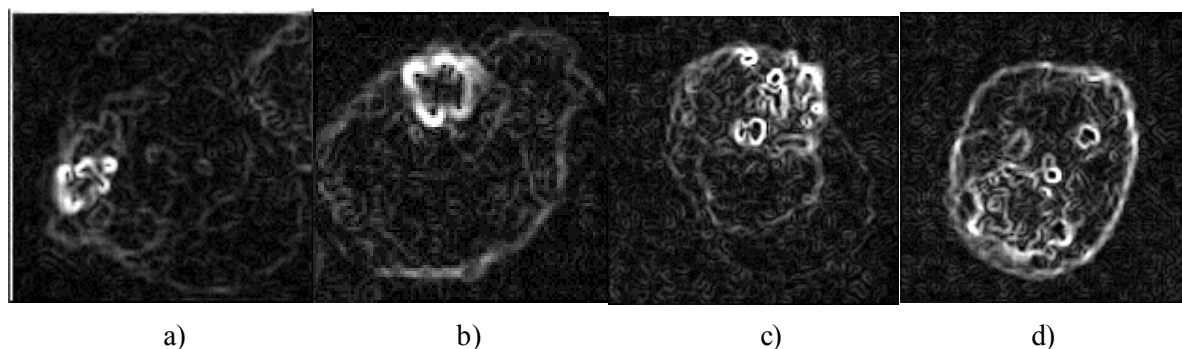


Fig. 4. TS₄PP localization at different times: a) 2h; b) 4 h; c) 8 h si d) 24 h

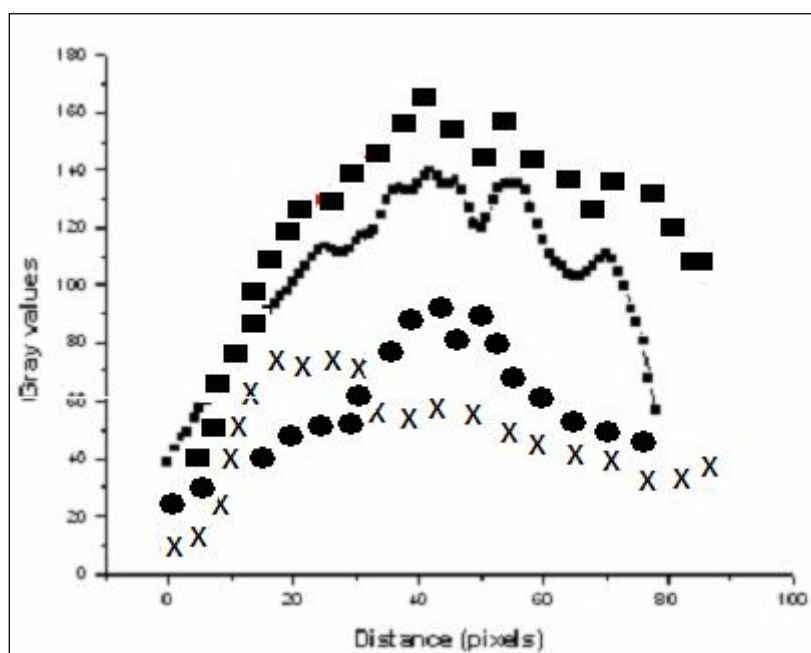


Fig. 5. Fluorescence intensity at different incubation time (x = 1h; ● = 4h; ---=8h; ■ = 24h)

Analysing this graphical representation, a significantly increasing of the fluorescence could be observed and in the same time an increasing of the occupied space between the cell membrane to nucleus.

All these data are a good proof of perinuclear aggregation of the sensitizer already recognized (Frackowiak et al., 2001). Also, these results are very correlated with the incubation time of TS₄PP

into K562 cells. As it is known there is a period of efficient accumulation of the photosensitizers in the tumoral tissue, Alexandrova et al. (2004) proved that is the reason why we have tested the time-dependency of the loading efficiency in K562 cells of the compounds. The yield of compound loading in K562 cell line was performed using flow cytometry measurements for time and cell concentration optimization. For all the compounds, the optimum incubation time is around 18-24h and it decreases after 48h. For TS₄PP compound the plateau of dependency is somehow extended after 24h of incubation. The optimum incubation time is in agreement with other photosensitizers used for loading into neoplastic cells lines. Less than 18h incubation time is probably insufficient for an optimum destructive effect of experimental PDT. For a longer incubation period, over 24h, the intracellular degradative processes are effective, destroying the accumulated photosensitizers and lowering the cell loading. We do not exclude although the cellular efflux mechanisms that over 24h of incubation can override the influx of the photosensitizer. The flow-cytometry studies showed that in the case of K562 cell line loading there is no population selection, thus on the whole, after a 24h period of incubation, the cell population has a maximum photosensitizer loading (maximum mean fluorescence) (Fig. 6).

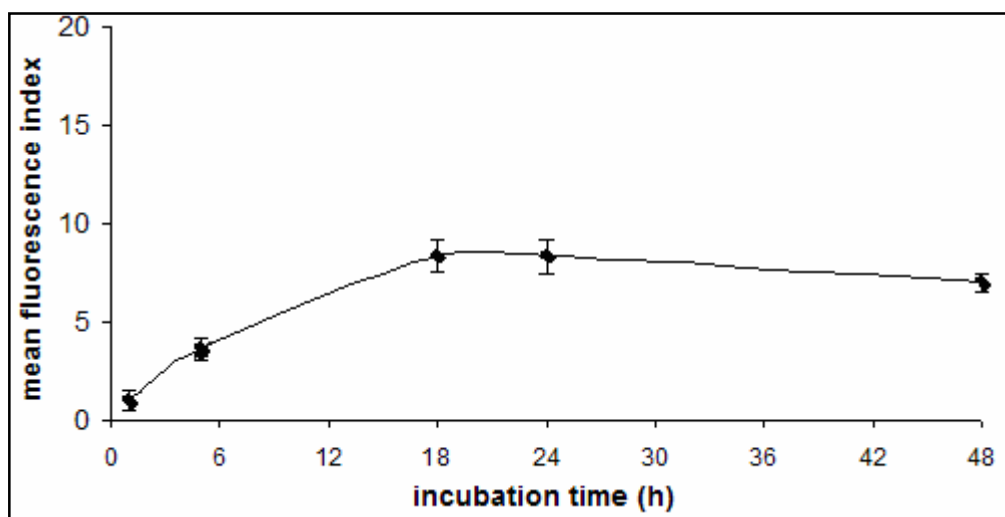


Fig. 6 Time of incubation dependency on the fluorescence of K562 cell line loaded with 20microg/mL TS₄PP

4. CONCLUSION

The present study demonstrates the non-uniform distribution of sensitizer TS₄PP, as evidenced by fluorescence microscopic studies. Because we measured the fluorescence at 650 nm, we presume that in the cells TS₄PP regain its monomeric form, although before incorporation the compound is in the J-aggregate form.

Even after 1 h of TS₄PP of administration, the sensitizer loading area is 45,29 % from cell surface, after 4h of administration, a sensitizer localization is observed in some cellular targets and an increased fluorescence intensity is observed. After 8 h and 24 h post-administration, the sensitizer is released from the cells, being recognized only in 5,79 % from total cell area.

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